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DEVELOPMENTALLY-REGULATED ENDOTHELIAL CELL LOCUS-1

[recitation of priority amended 3-11-2002]

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1. INTRODUCTION

The present invention relates to a member of a novel gene family referred to as developmentally-regulated endothelial cell locus-1 (*del-1*). In particular, the invention relates to *del-1* nucleotide sequences, Del-1 amino acid sequences, methods of expressing a functional gene product, antibodies specific for the gene product, and methods of using the gene and gene product. Since *del-1* is expressed in endothelial cells and certain cancer cells, it may be useful as an endothelial cell and tumor marker. In addition, the ability of Del-1 protein to inhibit vascular formation provides for its use as an anti-angiogenic agent.

2. BACKGROUND OF THE INVENTION

2.1. ENDOTHELIAL CELL BIOLOGY AND BLOOD VESSEL DEVELOPMENT

The endothelium occupies a pivotal position at the interface between the circulating humoral and cellular elements of the blood, and the solid tissues which constitute the various organs. In this unique position, endothelial cells regulate a large number of critical processes. Such processes include leukocyte adherence and transit through the blood vessel wall, local control of blood vessel tone, modulation of the immune response, the balance between thrombosis and thrombolysis, and new blood vessel development (Bevilacqua et al., 1993, *J. Clin. Invest* 91:379-387; Folkman et al., 1987, *Science* 235:442-447; Folkman et al., 1992, J. Biol. Chem. 267:10931-10934; Gimbrone, 1986, Churchill Livingstone, London; Issekutz, 1992, *Curr. Opin. Immunol.*

4:287-293; Janssens et al., 1992, *J. Biol. Chem.* 267:14519-14522; Lamas et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:6348-6352; Luscher et al., 1992, *Hypertension* 19:117-130; Williams et al., 1992, *Am. Rev. Respir. Dis.* 146:S45-S50; Yanagisawa, et al., 1988, *Nature* 332:411-415).

Endothelial cell dysfunction has been postulated as a central feature of vascular diseases such as hypertension and atherosclerosis. In this context, the ability of the endothelium to synthesize smooth muscle cell mitogens and factors which control smooth muscle contraction has received much attention (Janssens et al., 1992, *J. Biol. Chem.* 267:14519-14522; Lamas et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:6348-6352; Luscher et al., 1992, *Hypertension* 19:117-130; Raines et al., 1993, *Br. Heart J.* 69:S30-S37; Yanagisawa et al., 1988, *Nature* 332:411-415). The endothelial cell has also become the focus of attention in the study of diseases which are not primarily vascular in nature. Diverse disease processes such as adult respiratory distress syndrome, septic shock, solid tumor formation, tumor cell metastasis, rheumatoid arthritis, and transplant rejection are now understood to be related to normal or aberrant function of the endothelial cell. A rapidly increasing number of pharmacologic agents are being developed whose primary therapeutic action will be to alter endothelial cell function. In addition, recent attention on gene therapy has focused on the endothelial cell (Nabel et al., 1991, *J. Am. Coll. Cardiol.* 17:189B-194B). Transfer of genes into the endothelial cell may afford a therapeutic strategy for vascular disease, or the endothelium may serve simply as a convenient cellular factory for a missing blood borne factor. Hence, information regarding fundamental processes in the endothelial cell will aid the understanding of disease processes and allow more effective therapeutic strategies.

Studies from a number of laboratories have characterized the ability of the endothelial cell to dramatically alter basic activities in response to cytokines such as tumor necrosis factor (TNF)-alpha. TNF-alpha stimulation induces

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significant alterations in the production of vasoactive compounds such as nitric oxide and endothelin, increases surface stickiness toward various types of leukocytes, and modulates the expression of both pro- and anti-coagulant factors (Cotran et al., 1990, *J. Am. Soc. Nephrol.* 1:225-235; Mantovani et al., 1992, *FASEB J.* 6:2591-2599). In turn, endothelial cells have been shown to be an important source for the production of cytokines and hormones, including interleukin 1, 6 and 8 (Gimbrone et al., 1989, *Science* 246:1601-1603; Locksley et al. 1987, *J. Immunol.* 139:1891-1895; Loppnow et al., 1989, *Lymphokine. Res.* 8:293-299; Warner et al., 1987, *J. Immunol.* 139:1911-1917).

The ability of endothelial cells to produce granulocyte, granulocyte-macrophage, and macrophage colony stimulating factors has led to speculation that endothelial cells are an important facet of hematopoietic development (Broudy et al., 1987, *J. Immunol.* 139:464-468; Seelentag et al., 1987, *EMBO J.* 6:2261-2265). Early studies have provided the foundation for the cloning of a large number of "endothelial cell-specific" genes. Some of these include ICAM-1, ICAM-2, VCAM-1, ELAM-1, endothelin-1, constitutive endothelial cell nitric oxide synthetase, thrombomodulin, and the thrombin receptor (Bevilacqua et al., 1989, *Science* 243:1160-1165; Jackman et al., 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:8834-8838; Janssens et al., 1992, *J. Biol. Chem.* 267:14519-14522; Lamas et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:6348-6352; Osborn et al., 1989, *Cell* 59:1203-1211; Staunton et al., 1989, *Nature* 339:61-64; Staunton et al., 1988, *Cell* 52:925-933; Vu et al, 1991, *Cell* 64:1057-1068; Yanagisawa et al., 1988, *Nature* 332:411-415).

All blood vessels begin their existence as a capillary, composed of only endothelial cells. Much of the molecular research investigating the role of endothelial cells in blood vessel development has focused on this process in the adult organism, in association with pathological conditions. In these situations, new blood vessels are formed by budding and branching of existing vessels. This process, which depends

on endothelial cell division, has been termed angiogenesis. Research on this process has focused primarily on small proteins which are growth factors for endothelial cells (Folkman et al., 1987, *Science* 235:442-447; Folkman et al., 1992, *J. Biol. Chem.* 267:10931-10934). Sensitive bioassays for angiogenesis have allowed the characterization of a number of angiogenic factors, from both diseased and normal tissues. Members of the fibroblast growth factor (FGF) family, platelet-derived endothelial cell growth factor, and vascular endothelial cell growth factor (vascular permeability factor), are a few of the angiogenic factors which have been characterized (Folkman et al., 1987, *Science* 235:442-447; Folkman et al., 1992, *J. Biol. Chem.* 267:10931-10934; Ishikawa et al., 1989, *Nature* 338:557-562; Keck et al., 1989, *Science* 246:1309-1312; Leung et al., 1989, *Science* 246:1306-1309).

Such information has provided some insight into the study of blood vessel development in the embryo. Studies linking vascular development to an angiogenic factor have resulted in the work with vascular endothelial cell growth factor (VEGF). VEGF expression has been correlated in a temporal and spatial fashion with blood vessel development in the embryo (Breier et al., 1992, *Development* 114:521-532). A high affinity VEGF receptor, *flk-1*, has been shown to be expressed on the earliest endothelial cells in a parallel fashion (Millauer et al., 1993, *Cell* 72:835-846).

Blood vessels form by a combination of two primary processes. Some blood vessel growth depends on angiogenesis, in a process very similar to that associated with pathological conditions in the adult. For instance, the central nervous system depends solely on angiogenesis for development of its vascular supply (Noden, 1989, *Am. Rev. Respir. Dis.* 140:1097-1103; Risau et al., 1988, *EMBO J.* 7:959-962). A second process, vasculogenesis, depends on the incorporation of migratory individual endothelial cells (angioblasts) into the developing blood vessel. These angioblasts appear to be components of almost all mesoderm,

and are able to migrate in an invasive fashion throughout the embryo (Coffin et al., 1991, *Anat. Rec.* 231:383-395; Noden, 1989, *Am. Rev. Respir. Dis.* 140:1097-1103; Noden, 1991, *Development* 111:867-876). The precise origin of this cell, and the characteristics of its differentiation have not been defined.

Understanding of the molecular basis of endothelial cell differentiation in blood vessel development may allow manipulation of blood vessel growth for therapeutic benefit. The ability to suppress blood vessel growth may also provide therapeutic strategies for diseases such as solid tumors and diabetic retinopathy. On the other hand, diseases such as coronary artery disease may be treated through pharmacologic induction of directed blood vessel growth, through increasing collateral circulation in the coronary vascular bed. Both vascular diseases such as atherosclerosis and hypertension and nonvascular diseases which depend on the endothelial cell will benefit from a better understanding of endothelial cells.

2.2. EPIDERMAL GROWTH FACTOR-LIKE DOMAIN

Epidermal growth factor (EGF) stimulates growth of a variety of cell types. EGF-like domains have been found in a large number of extracellular and membrane bound proteins (Anderson, 1990, *Experientia* 46(1):2; and Doolittle, 1985, *TIBS*, June:233). These proteins include molecules that function as soluble secreted proteins, growth factors, transmembrane signal and receptor molecules, and components of the extracellular matrix (Lawler and Hynes, 1986, *J. Cell. Biol.* 103:1635; Durkin et al., 1988, *J. Cell Biol.* 107:2749; Wu et al., 1990, *Gene* 86:275; Bisgrove and Raff, 1993, *Develop. Biol.* 157:526;).

In many cases, multiple tandem repeats of a characteristic 40 amino acid long, 6 cysteine-containing sequence are observed (Anderson, 1990, *Experientia* 46(1):2). EGF-like domains are homologous to the peptide growth factor EGF which consists of a single copy of the standard EGF

domain. These domains have been highly conserved in evolution, being found in species as diverse as nematodes, Drosophila, sea urchins, and vertebrates.

The EGF molecule and the closely related transforming growth factor (TGF) alpha induce cell proliferation by binding to a tyrosine kinase receptor. It has been suggested that other EGF-like domains also function as ligands for receptor molecules (Engel, 1989, *FEBS Lett.* 251:1-7). Fundamentally, EGF repeats are protein structures that participate in specific protein-protein binding interactions.

The Drosophila Notch protein, the Nematode lin-12 and glp-1 proteins, and the closely related vertebrate homologs, Motch (mouse Notch), Xotch (Xenopus Notch), rat Notch, and TAN 1 (human Notch) are membrane bound receptor molecules that control the specification of cell fate for a variety of cell types early in embryogenesis (Rebay et al., 1991, *Cell* 67:687; Hutter and Schnabel, 1994, *Development* 120:2051; Del Amo et al 1992, *Development* 115:737; Reaume et al. 1992 *Develop. Biol.* 154:377; and Ellisen et al., 1991, *Cell* 66:649). Specific EGF-like repeats in the Notch receptors are binding sites that attach to protein ligands leading to signal transduction (Rebay et al., 1991 *Cell* 67:687; Couso and Arias, 1994, *Cell* 79:259; Fortini and Artavanis-Tsakonas, 1994, *Cell* 79:273; Henderson et al., 1994, *Development* 120:2913). Extracellular matrix proteins such as thrombospondin, entactin, tenascin and laminin play key roles in morphogenesis by providing the physical scaffold to which cells attach to form and maintain tissue morphologies (Frazier, 1987, *J. Cell. Biol.* 105:625; Taraboletti et al., 1990, *J. Cell. Biol.* 111:765; Ekblom et al., 1994, *Development* 120:2003).

2.3. DISCOIDIN I/FACTOR VIII-LIKE DOMAINS

A homologous domain structure has been discovered in coagulation factors VIII and V (Kane and Davie, 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:6800). This domain is related to a more ancient structure first observed in the discoidin I

protein produced by the cellular slime mold *Dictyostelium discoideum*. Discoidin I is a carbohydrate binding lectin secreted by Dictyostelium cells during the process of cellular aggregation and is involved in cell-substratum attachment and ordered cell migration (Springer et al., 1984, *Cell* 39:557).

Discoidin I/factor VIII-like domains have also been observed in a number of other proteins. For example, milk fat globule protein (BA46), milk fat globule membrane protein (MFG-E8), breast cell carcinoma discoidin domain receptor (DDR), and the Xenopus neuronal recognition molecule (A5) (Stubbs et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.* 87:8417; Larocca et al., 1991, *Cancer Res.* 51:4994; Johnson et al., 1993, *Proc. Natl. Acad. Sci. U.S.A.* 90:5677). The discoidin I/factor VIII-like domains of the vertebrate proteins are all distantly related to the Dictyostelium sequence but more closely related to each other.

Discoidin I/factor VIII-like domains are rich in positively charged basic amino acids and are believed to bind to negatively charged substrates such as anionic phospholipids or proteoglycans. Both of the milk fat globule proteins have been shown to associate closely with cell membranes and the coagulation factors VIII and V interact with specific platelet membrane proteins (Stubbs et al., 1990 *Proc. Nat. Acad. Sci. U.S.A.* 87:8417; Larocca et al., 1991, *Cancer Res.* 51:4994).

3. SUMMARY OF THE INVENTION

The present invention relates to a novel gene family referred to as *del-1*. In particular, it relates to *del-1* nucleotide sequences, expression vectors containing the sequences, genetically-engineered host cells expressing *del-1*, Del-1 protein, Del-1 mutant polypeptides, methods of expressing *del-1* and methods of using *del-1* and its gene product in various normal and disease conditions such as cancer.

The invention is based, in part, upon Applicants' isolation of a murine DNA clone (SEQ ID NO: 9), *del-1*, and its homologous human counterpart (SEQ ID NO: 11). Structural features of the Del-1 protein are deduced by homology comparisons with sequences in the Genbank and NBRF-PIR databases. The protein is a modular molecule composed of repeats of two different sequence motifs which are present in a number of distinct proteins. The two sequence motifs are known as the EGF-like domain (SEQ ID NO: 26) and the discoidin I/factor VIII-like domain (SEQ ID NOS: 1-8). These domains are defined by characteristic patterns of conserved amino acids distributed throughout the molecule at specific locations. While Del-1 shows certain sequence homology with other proteins, it is unique in both its primary sequence and its overall structure. In all cases in which EGF-like and discoidin I-like domains have been identified, both of these structures are always found in extracellular locations. Variant forms of Del-1 protein exist, and one form is shown herein to be an extracellular matrix protein and is associated with the cell surface. The expression pattern of *del-1* further indicates that it is involved in endothelial cell function. In addition, a number of human tumor cells express *del-1*. Furthermore, host-derived blood vessels that traverse the tumor nodule also express *del-1*. The Del-1 protein inhibits vascular morphogenesis and binds to $\alpha V\beta 3$ as its cellular receptor. Therefore, a wide variety of uses are encompassed by the present invention, including but not limited to, the use of Del-1 as a tumor marker for cancer diagnosis and treatment, the isolation of embryonic endothelial cells, the identification of Del-1 binding partners, and the stimulation or inhibition of endothelial cell growth and blood vessel formation.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Genomic organization of 42 kb of the murine *del-1* locus, as characterized by cloning from a λfix library constructed

from the SLM275 transgenic mouse, and a wildtype 129SV λfix library. The dashed line indicates DNA studied to date by zoo blot and exon trapping. The location of the exon identified by exon trapping is shown.

Figure 2. Homology analysis between the deduced amino acid sequence of the putative *del-1* gene (m-del1) (SEQ ID NO: 1) and other proteins with "discoidin-like domains." Identical residues are boxed, conserved residues are ~~shaded~~ in bold (previously amended) (Geneworks, Intelligenetics, Mountain View, CA). m-del-1 sequence (SEQ ID NO: 1) was derived from a trapped exon and mouse embryo cDNAs. Abbreviations:h-MFG, human milk fat globule protein (SEQ ID NO: 2); h-FV, human coagulation factor V (SEQ ID NO: 3); m-FVIII, mouse coagulation factor VIII (SEQ ID NO: 4); X-A5b1 (SEQ ID NO: 5) and X-A5b2 (SEQ ID NO: 6), b1 and b2 domains of Xenopus neuronal antigen A5; dis-I, discoidin I (SEQ ID NO: 7); consensus sequence (SEQ ID NO: 8). (amended 3.11.2002)

Figure 3A-~~3E~~ 3D Nucleotide sequence and deduced amino acid sequence of murine *del-1* cDNA (SEQ ID NO: 9) and (SEQ ID NO: 10).

Figure 4A-4C. Nucleotide sequence and deduced amino acid sequence of human *del-1* cDNA (SEQ ID NO: 11) and (SEQ ID NO: 14).

Figure 5. Murine *del-1* fragment (SEQ ID NO: 19) used as probe for human *del-1* cloning and Northern blot analysis.

Figure 6. Amino acid sequence comparison between murine (m-del-1) (SEQ ID NO: 10) and human (h-del-1) (SEQ ID NO: 30 ~~29~~) Del-1 proteins. The EGF-like and discoidin-like domains are indicated by "egf" and "discoidin," respectively.

Figure 7. The small rectangles labeled "EGF" show the location and relative sizes of the three EGF-like domains of Del-1. These regions of the protein are approximately 40 amino acids long. Each EGF-like domain contains six cysteine residues and additional conserved amino acids, distributed in a pattern which is highly conserved among proteins that contain this common motif. In addition, the amino acid sequence RGD occurs in the center of the second EGF-like repeat. This sequence is found in a variety of extracellular matrix proteins and, in some cases, it is required for binding to integrin proteins. An RGD sequence is present in the same position in the second EGF-like repeat of MFG-E8.
The large rectangles on the right side represent tandem discoidin I/factor VIII-like domains. This protein motif is based on a conserved pattern of amino acids defined by the homology between the *D. discoidium* discoidin I protein and mammalian coagulation factor VIII.

Figure 8. The 54.2% amino acid homology between human Del-1 (SEQ ID NO. 21) and MFG-E8 (SEQ ID NO: 20 ~~21~~) in the tandem discoidin I/factor VIII domains is shown. These domains are rich in the basic amino acids arginine and lysine. The 5'

domain contains 12 arginines and 12 lysines versus 9 acidic residues, while the 3' domain contains 8 arginines and 10 lysines versus 16 acidic residues. A similar domain in the coagulation factor VIII protein is believed to bind to negatively charged phospholipids on the surface of platelets. The MFG-E8 protein has been found to associate tightly with milk fat globule membranes.

Figure 9. The predicted amino acid sequence at the amino terminus of the human Del-1 protein (SEQ ID NO: 22) shows characteristics common to signal peptides. The putative signal, begins with a basic arginine residue and is followed by a stretch of 18 amino acids rich in hydrophobic residues. Signal peptides typically end with a small amino acid such as glycine or alanine. In addition, the Chou and Fasman algorithm predicts that the putative signal sequence is followed by a protein turn structure, a feature commonly found after signal peptides. The Del-1 protein is secreted by expressing cells.

Figure 10. Sequence similarities between the three EGF-like domains of Del-1, ~~(SEQ ID NOS: 23-25)~~ and homology with the consensus EGF-like domain amino acid sequence (SEQ ID NO: 26). Also, the amino acid sequence RGD is in the center of the second EGF-like repeat. This sequence is found in a variety of extracellular matrix proteins and, in some cases is required for binding to integrin proteins. An RGD sequence is present in the

same position in the second EGF-like repeat of MFG-E8.

Figure 11. Human *del-1* splicing variant partial sequence ~~(SEQ ID NO: 27)~~ showing the variation as compared with the major form (SEQ ID NO:30).

(Nucleotide sequence: SEQ ID NO:27; amino acid sequence: SEQ ID NO. 31)

Figure 12A-12E. Murine *del-1* truncated minor nucleotide and deduced amino acid sequences (SEQ ID NO: 28) and (SEQ ID NO: 29).

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Figure 13A-13H. X-gal staining in whole mount and tissue sections of embryos from the SLM275 line. (13A) Embryo at 7.5 days pc (headfold stage) stained as whole mount. X-gal staining is seen in cells of the extraembryonic mesoderm (xm) which will give rise to the yolk sac and associated blood islands. Abbreviations: ng, neural groove. Photographed at 70x. (13B) Section of yolk sac blood islands from 8 day pc embryo stained as a whole mount with membranes intact and subsequently sectioned and counterstained. Clusters of round cells in the blood islands show X-gal staining (arrow), while mature endothelial cells do not stain (open arrowhead). Photographed at 400x. (13C) Embryo at 9.5 days pc. Prominent X-gal staining (blue-green) is seen in the heart and outflow tract (mid-portion of embryo). In addition, the aorta (arrowhead) and intervertebral vessels are stained. Photographed at approximately 30x, darkfield illumination. (13D) Section of 9.5 day embryo showing heart and outflow tract. This section indicates that X-gal staining in the heart and outflow tract is

restricted to the endothelial cells (endocardium). Section was counterstained with hematoxylin and eosin, photographed at 200x. (13E) Embryo at 13.5 days pc, dissected and X-gal stained as a whole mount. At this stage, as confirmed by study of tissue sections, endothelial cells lining the ventricle (v) and large vessels such as the aorta (filled arrowhead) have lost most of their staining. Staining of the endothelial cells of the atrium (a) has diminished but is still apparent in the whole mount. Most pronounced at this stage is staining in the developing lungs (open arrowheads). X-gal staining cells are clearly associated with the glandular buds of the lung, but it is not possible to identify these cells in the whole mount. The only non-cardiovascular cells which exhibit X-gal staining are cells in the regions of ossification, such as in the proximal ribs shown here. Photographed at 50x. (13F) Embryo at 13.5 days, stained as whole mount, sectioned, counterstained with nuclear fast red. X-gal staining in lung tissue shown here is associated with endothelial cells, as seen in vascular channels cut in transverse (arrow) and longitudinal (arrowhead) planes. Staining is not associated with bronchial cells. Section was photographed at 400x. (13G) Cross-section through a valve forming in the outflow tract of a 13.5 day embryo. Endothelial cells in blood vessel wall are undergoing an epithelial-mesenchymal transformation, leading to formation of the valve tissue. Stained cells are seen within

the forming valve structure, indicating that these cells continue to express the *del-1* marker during this phenotypic transformation. The embryo was stained as a whole mount, sectioned, counterstained with nuclear fast red and photographed at 400x. (13H) Spiral septal formation in the outflow tract of the heart at 9.5 days pc. Endothelial cells are undergoing an epithelial-mesenchymal transformation, becoming mesenchymal in morphology and behavior. Endothelial cells continue to express the transgene marker for some time after this transformation. Section from whole mount stained embryo, 200x.

Figure 14A & 14B. Immunoblotting employing *del-1* transfected yolk sac cells. (14A) Yolk sac YS-B cells stably transfected with a eukaryotic expression vector encoding the murine major form of *del-1*(+), or an empty expression vector(-) were selected and evaluated as pools for expression of Del-1 protein. Protein was isolated from cells lysed in cell lysis buffer (Lysis) or standard Laemmli gel loading buffer (Laemmli), or from the extracellular matrix remaining after transfected cells were removed from the culture dish (ECM). The dominant band corresponds to a molecular weight of 52 kilodaltons (kDa). Lower molecular weight bands most likely represent protein degradation products, although the use of alternative translation initiation sites is also possible. (14B) YS-B cells were stably transfected with the *del-1* expression construct, or the empty expression plasmid,

and selected as individual clones. Clones expressing *del-1* were selected for varying levels of protein production, as assayed by western blot analysis of extracellular matrix protein. Clone L10 shows the highest level of *del-1* mRNA, clones L13 and L14 have an intermediate amount of message, and a negative control clone does not express *del-1*.

Figure 15A-15B. Immunostaining of yolk sac cells. (15A) *del-1* transfected yolk sac cells and the extracellular matrix are stained with anti-Del-1 antibody. The arrows indicate cell membrane staining. (15B) Mock-transfected yolk sac cells are not stained with antibody.

Figure 16. Immunostaining of Del-1 in the developing bone (vertebral column) of a 13.5 day mouse embryo. The ~~laquanae~~ lacunae within the bone are structures composed of extracellular matrix proteins and they are stained for Del-1.

Figure 17. Immunostaining of human glioma grown in nude mice. (17A) tumor cells are stained with anti-Del-1 antibody. Polarized staining pattern is observed (arrows). (17B) a blood vessel is stained with anti-Del-1 within the tumor.

Figure 18A-18H. (18A) The parental yolk sac cell line YS-B under routine culture conditions. Phase contrast, photo 100x. (18B) YS-B cells after 24 hrs on "MATRIGEL" show a pattern of vascular morphogenesis. Cells were stained with toluidine blue. Brightfield, photo

40x. (18C) Negative control transfectants form a vascular network on "MATRIGEL" after 24 hours. Light areas represent organized cells; photographed under dark field illumination at 50x. (18D) Yolk sac transfectant, clone L10, after 24 hrs on "MATRIGEL" shows no evidence of vascular formation, cells instead produce numerous aggregates. Darkfield illumination, photo 50x. (18E) Parental yolk sac YS-B cells grown on a matrix produced by negative control transfectants make a complex structural network. Light areas represent organized cells; photographed under dark field illumination at 30x. (18F) Parental YS-B cells grown on a matrix produced by *del-1* transfectants. Cells are forming a dense monolayer, with no evidence of organization. Photographed under darkfield illumination at 30x. (18G) Aggregates of negative control transfected yolk sac cells are placed onto polymerized "MATRIGEL". After 24 hrs, cells show sprouting angiogenesis. Photographed under phase contrast, at 100x. (18H) Aggregates of *del-1* transfected yolk sac clone L10 are placed onto polymerized "MATRIGEL" as in 18G. Photographed after 24 hrs (100x), these cells show no evidence of sprouting.

Figure 19. The binding of murine recombinant Del-1 to HUVEC is inhibited by an anti-$\alpha V\beta 3$ antibody. The relative cell number of HUVEC adhered to plates coated with recombinant Del-1 is shown in the presence of various antibodies.

Figure 20. The binding of murine recombinant Del-1 to HUVEC is inhibited by RGD peptides. The relative cell number of HUVEC adhered to plates coated with recombinant Del-1 is shown in the presence of RGD and RGE peptides at 10 μg/ml.

Figure 21A & 21B Two ideograms illustrating the chromosomal position of P1 clone 10043 at 5q14. (21A) nomenclature for human chromosomes adopted from the International System for Human Cytogenetic Nomenclature (1985). (21B) an ideogram adopted from *Cytogenet. Cell Genet.* 65:206-219 (1994) which shows the relative band positions and arm ratios derived from actual chromosome measurements.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel family of genes herein referred to as *del-1*. Described below are methods for cloning members of this gene family, characteristics of a murine member and its human homolog, expression of recombinant gene products, and methods of using the gene and its gene product. Structurally, members of this gene family contain three EGF-like domains and two discoidin I/factor VIII-like domains.

The overall structure of the *del-1* molecule is similar to the milk fat globule membrane protein (MFG-E8) (Stubbs et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:8417). MFG-E8 is highly expressed by a large portion of human breast tumors as well as by lactating mammary epithelial cells. It consists of two tandem EGF-like domains followed by two discoidin I/factor VIII-like domains. The function of MFG-E8 is not known but it has been shown to associate closely with cell membranes and has been investigated as a target for antibody-based tumor imaging techniques. The observed association of MFG-E8 with cell membranes indicates the potential use of

antibodies against Del-1 to identify and sort endothelial cells from mixed cell populations, and to target tumor cells that express Del-1 for diagnosis and therapy.

The second EGF-like repeat of MFG-E8 contains the amino acid sequence arg-gly-asp (RGD) in the same position as the second EGF-like repeat of Del-1. The RGD sequence has been shown to be a cell binding site for fibronectin, discoidin I, nidogen/entactin, and tenascin (Anderson, 1990, *Experientia* 46:2). The binding of fibronectin to cell surface integrin molecules through the RGD sequence has been extensively studied (Main et al., 1992, *Cell* 71:671; Hynes, 1992, *Cell* 69:11). Integrins appear to be the major receptors by which cells attach to extracellular matrices. Substrate binding to integrins has been shown to initiate signal transduction leading to events such as tyrosine phosphorylation, cytoplasmic alkalinization, activation of secretion and differentiation (Hynes, 1992, *Cell* 69:11). The presence of the RGD sequence in Del-1 indicates that this portion of the molecule may bind cell surface integrins, possibly triggering certain developmental events. In particular, Del-1 is shown to bind to integrin $\alpha V\beta 3$ on endothelial cells. In several cases, synthetic peptides containing the RGD sequence have been shown to compete with native protein for integrin binding and prevent the initiation of downstream events (Brooks et al., 1994, Cell 79:1157).

For clarity of discussion, the invention is described in the subsections below by way of example for the *del-1* genes and their products in mice and in humans. However, the findings disclosed herein may be analogously applied to other members of the *del-1* family in all species.

5.1. THE *DEL-1* CODING SEQUENCE

The present invention relates to nucleic acid molecules and polypeptides of the *del-1* gene family. In a specific embodiment by way of example in Section 6, infra, murine and human *del-1* nucleic acid molecules were cloned, and their nucleotide and deduced amino acid sequences characterized.

Both the nucleotide coding sequence and deduced amino acid sequence of *del-1* are unique. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the *del-1* gene product can be used to generate recombinant molecules which direct the expression of *del-1* gene.

Enhancer trapping is a strategy which has been successfully employed in genetic analysis in Drosophila but is also applicable to higher organisms. This method identifies regulatory regions in genomic loci through their influence on reporter genes (Okane et al., 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:9123-9127). The reporter gene, as a transcriptional unit under the control of a weak constitutively expressed eukaryotic promoter, is introduced into a large number of organisms. The offsprings of these organisms are then screened by analysis of the pattern of reporter gene expression. Lines which show expression in the appropriate cells at the appropriate time are maintained for further study. This strategy has successfully identified a number of loci in Drosophila involved in complex developmental processes.

Enhancer trap experiments have been employed in mice to a limited extent (Allen et al., 1988, *Nature* 333:852-855). A number of such experiments were through fortuitous integration of a reporter gene into a locus of interest (Kothary et al., 1988, *Nature* 335:435-437). Using this method coupled with genomic and cDNA cloning, the murine *del-1* locus associated with the transgene was identified. A genomic library is generated from the transgenic mouse, and a probe from the transgene used to isolate clones containing the transgene and sequences flanking the integration site. Characterization of the regulatory region is accomplished by employing flanking sequences in functional assays, via transfection experiments with an appropriate cell culture line, or via further transgenic experiments (Bhat et al., 1988, *Mol. Cell. Biol.* 8:3251-3259).

For analysis of the transcription unit, it is necessary to identify a region of flanking sequence which contains a portion of exon. This has been accomplished by blindly using flanking genomic sequences as probes in northern blots or zoo blots (Soinen et al., 1992, *Mechanisms of Development* 39:111-123). DNA fragments thus identified to contain exon sequence are employed as probes for cDNA cloning. Similar cloning experiments have been conducted to characterize loci inactivated by insertional mutagenesis associated with transgene integration. These experiments indicate that deletions of large regions of genomic DNA may accompany transgene integration, and that complexity of the transcription unit may greatly complicate this type of analysis (Karls et al., 1992, *Mol. Cell. Biol.* 12:3644-3652; Woychik et al., 1990, *Nature* 346:850-853).

Subsequent analysis of the *del-1* sequence has revealed both EGF-like and discoidin I/factor VIII-like domains. The shared homology between *del-1* and other known molecules is discussed in Section 6.2, *infra*. However, this molecule also contains regions of previously unreported unique nucleotide sequences. Northern blot hybridization analysis indicates that *del-1* mRNA is highly expressed in fetal cells. In addition, the *del-1* sequence is expressed in certain tumor cells.

In order to clone the full length cDNA sequence from any species encoding the entire *del-1* cDNA or to clone variant forms of the molecule, labeled DNA probes made from nucleic acid fragments corresponding to any murine and human of the partial cDNA disclosed herein may be used to screen a cDNA library. More specifically, oligonucleotides corresponding to either the 5' or 3' terminus of the cDNA sequence may be used to obtain longer nucleotide sequences. Briefly, the library may be plated out to yield a maximum of 30,000 pfu for each 150 mm plate. Approximately 40 plates may be screened. The plates are incubated at 37°C until the plaques reach a diameter of 0.25 mm or are just beginning to make contact with one another (3-8 hours). Nylon filters are

placed onto the soft top agarose and after 60 seconds, the filters are peeled off and floated on a DNA denaturing solution consisting of 0.4N sodium hydroxide. The filters are then immersed in neutralizing solution consisting of 1M Tris HCL, pH 7.5, before being allowed to air dry. The filters are prehybridized in casein hybridization buffer containing 10% dextran sulfate, 0.5M NaCl, 50mM Tris HCL, pH 7.5, 0.1% sodium pyrophosphate, 1% casein, 1% SDS, and denatured salmon sperm DNA at 0.5 mg/ml for 6 hours at 60°C. The radiolabelled probe is then denatured by heating to 95°C for 2 minutes and then added to the prehybridization solution containing the filters. The filters are hybridized at 60°C for 16 hours. The filters are then washed in 1X wash mix (10X wash mix contains 3M NaCl, 0.6M Tris base, and 0.02M EDTA) twice for 5 minutes each at room temperature, then in 1X wash mix containing 1% SDS at 60°C for 30 minutes, and finally in 0.3X wash mix containing 0.1% SDS at 60°C for 30 minutes. The filters are then air dried and exposed to x-ray film for autoradiography. After developing, the film is aligned with the filters to select a positive plaque. If a single, isolated positive plaque cannot be obtained, the agar plug containing the plaques will be removed and placed in lambda dilution buffer containing 0.1M NaCl, 0.01M magnesium sulfate, 0.035M Tris HCl, pH 7.5, 0.01% gelatin. The phage may then be replated and rescreened to obtain single, well isolated positive plaques. Positive plaques may be isolated and the cDNA clones sequenced using primers based on the known cDNA sequence. This step may be repeated until a full length cDNA is obtained.

It may be necessary to screen multiple cDNA libraries from different tissues to obtain a full length cDNA. In the event that it is difficult to identify cDNA clones encoding the complete 5' terminal coding region, an often encountered situation in cDNA cloning, the RACE (Rapid Amplification of cDNA Ends) technique may be used. RACE is a proven PCR-based strategy for amplifying the 5' end of incomplete cDNAs. 5'-RACE-Ready cDNA synthesized from human fetal liver containing

a unique anchor sequence is commercially available (Clontech). To obtain the 5' end of the cDNA, PCR is carried out on 5'-RACE-Ready cDNA using the provided anchor primer and the 3' primer. A secondary PCR reaction is then carried out using the anchored primer and a nested 3' primer according to the manufacturer's instructions. Once obtained, the full length cDNA sequence may be translated into amino acid sequence and examined for certain landmarks such as a continuous open reading frame flanked by translation initiation and termination sites, EGF-like domain, discoidin I-like domain, a potential signal sequence and transmembrane domain, and finally overall structural similarity to the *del-1* genes disclosed herein.

5.2. EXPRESSION OF *DEL-1* SEQUENCE

In accordance with the invention, a *del-1* polynucleotide sequence which encodes the Del-1 protein, mutant polypeptides, peptide fragments of Del-1, Del-1 fusion proteins or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of Del-1 protein, Del-1 peptide fragments, fusion proteins or a functional equivalent thereof, in appropriate host cells. Such *del-1* polynucleotide sequences, as well as other polynucleotides which selectively hybridize to at least a part of such *del-1* polynucleotides or their complements, may also be used in nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the Del-1 protein. Such DNA sequences include those which are capable of hybridizing to the murine and/or human *del-1* sequences under stringent conditions. The phrase "stringent conditions" as used herein refers to those hybridizing conditions that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M

sodium citrate/0.1% SDS at 50°C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide, with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within a Del-1 sequence, which result in a silent change thus producing a functionally equivalent Del-1 protein. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine, histidine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: glycine, asparagine, glutamine, serine, threonine, tyrosine; and amino acids with nonpolar head groups include alanine, valine, isoleucine, leucine, phenylalanine, proline, methionine, tryptophan.

The DNA sequences of the invention may be engineered in order to alter a *del-1* coding sequence for a variety of ends, including but not limited to, alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, *e.g.*, site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc.

Based on the domain organization of the Del-1 protein, a large number of Del-1 mutant polypeptides can be constructed by rearranging the nucleotide sequences that encode the Del-1 domains. Since the EGF-like domains of Del-1 are known to be involved in protein binding, Del-1 may directly bind to other cell surface receptors or extracellular matrix proteins via these domains, thereby controlling cell fate determination or differentiation in a manner similar to Notch and Notch ligands. Additionally, the RGD sequence in the second EGF-like domain is known to bind to certain integrins, thus Del-1 may regulate cell adhesiveness, migration, differentiation and viability via this sequence. The discoidin I-like domains of Del-1 are involved in a separate type of cell binding activity. In accordance with the observed properties of Factors V and VIII, Del-1 may directly bind proteoglycans in the extracellular matrix or on the cell surface via those domains. Therefore, the combination of various domains of full-length Del-1 permits the molecule to perform diverse types of binding. For example, the major form of Del-1 may be able to cluster integrin receptors by way of both EGF-like and discoidin I-like domains. In contrast, smaller fragments of Del-1 or its minor form would bind integrins without the ability to induce receptor clustering, and thus induce alternative signals to cells.

In view of the foregoing, the Del-1 mutant polypeptides can be generated and their functional activities compared. In addition to the minor form, Del-1 mutants may be constructed to contain only the EGF-like or discoidin I-like domains. Additionally, smaller polypeptides can be made from constructs that contain any one of the EGF-like and discoidin I-like domains.

In another embodiment of the invention, a *del-1* or a modified *del-1* sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for molecules that bind Del-1, it may be useful to encode a chimeric Del-1 protein expressing a heterologous epitope that is recognized by a

commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between a Del-1 sequence and the heterologous protein sequence, so that the Del-1 may be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence of Del-1 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers *et al.*, 1980, *Nuc. Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 180, *Nuc. Acids Res. 9(10)*:2331; Matteucci and Caruthers, 1980, *Tetrahedron Letter 21*:719; and Chow and Kempe, 1981, *Nuc. Acids Res. 9(12)*:2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize an Del-1 amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (*e.g.*, see Creighton, 1983, *Proteins Structures And Molecular Principles*, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; see Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and Co., N.Y., pp. 34-49).

In order to express a biologically active Del-1, the nucleotide sequence coding for Del-1, or a functional equivalent, is inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The *del-1* gene products as well as host cells or cell lines transfected or transformed with recombinant *del-1* expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (*i.e.*, monoclonal or polyclonal) that competitively inhibit activity of Del-1 protein and neutralize its activity; and antibodies that mimic the activity of Del-1 binding partners such as a receptor. Anti-Del-1 antibodies may be used in detecting and quantifying expression of Del-1 levels in cells

and tissues such as endothelial cells and certain tumor cells, as well as isolating Del-1-positive cells.

5.3. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the *del-1* coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the *del-1* coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the *del-1* coding sequence; yeast transformed with recombinant yeast expression vectors containing the *del-1* coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the *del-1* coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the *del-1* coding sequence; or animal cell systems. The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter; cytomegalovirus promoter) and the like may be used; when cloning in insect

cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll α/β binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the *del-1* DNA, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the *del-1* expressed. For example, when large quantities of *del-1* are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the *del-1* coding sequence may be ligated into the vector in frame with the *lacZ* coding region so that a hybrid AS-*lacZ* protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety. In particular, murine *del-1* major and minor coding sequences have been

inserted in pET28a (Novagen Inc.) which contains a T7 promoter, and pMALC2 (New England Biolabs). These vectors encode fusion proteins which can be readily purified.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used, the expression of the *del-1* coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

An alternative expression system which could be used to express *del-1* is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is

used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The *del-1* coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the *del-1* coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (e.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051). A commercially available baculovirus expression vector pFastBac 1 (Gibco BRL, Inc.) has been constructed to contain the murine *del-1* coding sequence.

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the *del-1* coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing *del-1* in infected hosts. (*e.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79:4927-4931).

Additionally, both the murine *del-1* and human coding sequences have been inserted in a mammalian expression vector, pcDNA3 (Invitrogen, Inc.), which is under the control of the cytomegalovirus promoter. Regulatable expression vectors such as the tetracycline inducible vectors may also

be used to express the coding sequences in a controlled fashion.

Specific initiation signals may also be required for efficient translation of inserted *del-1* coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire *del-1* gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the *del-1* coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the *del-1* coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. The presence of several consensus N-glycosylation sites in the *del-1* extracellular domain support the possibility that proper modification may be important for Del-1 function. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such

mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, yolk sac cells, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the *del-1* may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the *del-1* DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the Del-1 protein on the cell surface. Such engineered cell lines are particularly useful in screening for molecules or drugs that affect *del-1* function.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and

hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and *ODC* (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

5.4. IDENTIFICATION OF CELLS THAT EXPRESS *DEL-1*

The host cells which contain the coding sequence and which express a biologically active *del-1* gene product or fragments thereof may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of *del-1* mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity. Prior to the identification of gene expression, the host cells may be first mutagenized in an effort to increase the level of expression of *del-1*, especially in cell lines that produce low amounts of *del-1*.

In the first approach, the presence of the *del-1* coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the *del-1* coding sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the *del-1* coding sequence is inserted within a marker gene

sequence of the vector, recombinants containing the *del-1* coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the *del-1* sequence under the control of the same or different promoter used to control the expression of the *del-1* coding sequence. Expression of the marker in response to induction or selection indicates expression of the *del-1* coding sequence.

In the third approach, transcriptional activity for the *del-1* coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the *del-1* coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes. Additionally, RT-PCR may be used to detect low levels of gene expression.

In the fourth approach, the expression of the Del-1 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. This can be achieved by using an anti-Del-1 antibody and a Del-1 binding partner such as $\alpha V\beta 3$. Alternatively, the biologic activities of Del-1 can be determmined by assaying its ability to inhibit vascular morphogenesis of endothelial cells.

5.5. USES OF *DEL-1* ENGINEERED CELL LINES

In an embodiment of the invention, the Del-1 protein and/or cell lines that express Del-1 may be used to screen for antibodies, peptides, small molecules natural and synthetic compounds or other cell bound or soluble molecules that bind to the Del-1 protein. For example, anti-Del-1 antibodies may be used to inhibit or stimulate Del-1 function. Alternatively, screening of peptide libraries with recombinantly expressed soluble Del-1 protein or cell lines expressing Del-1 protein may be useful for identification of therapeutic molecules that function by inhibiting or

stimulating the biological activity of Del-1. The uses of the Del-1 protein and engineered cell lines, described in the subsections below, may be employed equally well for other members of the *del-1* gene family in various species.

In an embodiment of the invention, engineered cell lines which express most of the *del-1* coding region or a portion of it fused to another molecule such as the immunoglobulin constant region (Hollenbaugh and Aruffo, 1992, Current Protocols in Immunology, Unit 10.19; Aruffo et al., 1990, Cell 61:1303) may be utilized to produce a soluble molecule to screen and identify its binding partners. The soluble protein or fusion protein may be used to identify such a molecule in binding assays, affinity chromatography, immunoprecipitation, Western blot, and the like. Alternatively, portions of *del-1* may be fused to the coding sequence of the EGF receptor transmembrane and cytoplasmic regions. Assuming that Del-1 can function as a cell-bound receptor, this approach provides for the use of the EGF receptor signal transduction pathway as a means for detecting molecules that bind to Del-1 in a manner capable of triggering an intracellular signal. On the other hand, Del-1 may be used as a soluble factor in binding to cell lines that express specific known receptors such as integrins. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in assays that are well known in the art.

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that stimulate or inhibit the biological activity of receptors through their interactions with the given receptor.

Identification of molecules that are able to bind to the Del-1 protein may be accomplished by screening a peptide library with recombinant soluble Del-1 protein. Methods for expression and purification of Del-1 are described in Section 5.2, *supra*, and may be used to express recombinant full length *del-1* or fragments of *del-1* depending on the functional domains of interest. For example, the EGF-like and discoidin I/factor VIII domains of *del-1* may be separately expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase support that interacts and forms a complex with Del-1, it is necessary to label or "tag" the Del-1 molecule. The Del-1 protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label to Del-1 may be performed using techniques that are well known in the art. Alternatively, *del-1* expression vectors may be engineered to express a chimeric Del-1 protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes or colored or magnetic beads.

The "tagged" Del-1 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between Del-1 and peptide species within the library. The library is then washed to remove any unbound protein. If Del-1 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diaminobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-Del-1 complex changes color, and can be easily identified and isolated physically under a dissecting microscope with a

micromanipulator. If a fluorescent tagged Del-1 molecule has been used, complexes may be isolated by fluorescence activated sorting. If a chimeric Del-1 protein expressing a heterologous epitope has been used, detection of the peptide/Del-1 complex may be accomplished by using a labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble Del-1 molecules, in another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the cell membrane to be functional. Methods for generating cell lines expressing *del-1* are described in Section 5.3. The cells used in this technique may be either live or fixed cells. The cells may be incubated with the random peptide library and bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where label or "tag" can be attached.

Various procedures known in the art may be used for the production of antibodies to epitopes of the natural and recombinantly produced Del-1 protein. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the ligand binding site of the Del-1 protein are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind Del-1 may be radioactively labeled allowing one to follow their location and distribution in the body after injection. Radioisotope tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* cells of tumors and metastases.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity Del-1 specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diphtheria toxin, ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The hybrid antibodies may be used to specifically eliminate Del-1 expressing tumor cells.

For the production of antibodies, various host animals may be immunized by injection with the recombinant or naturally purified Del-1 protein, fusion protein or peptides, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to Del-1 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy,

Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce Del-1-specific single chain antibodies.

Hybridomas may be screened using enzyme-linked immunosorbent assays (ELISA) in order to detect cultures secreting antibodies specific for refolded recombinant Del-1. Cultures may also be screened by ELISA to identify those cultures secreting antibodies specific for mammalian-produced Del-1. Confirmation of antibody specificity may be obtained by western blot using the same antigens. Subsequent ELISA testing may use recombinant Del-1 fragments to identify the specific portion of the Del-1 molecule with which a monoclonal antibody binds. Additional testing may be used to identify monoclonal antibodies with desired functional characteristics such as staining of histological sections, immunoprecipitation of Del-1, or neutralization of Del-1 activity. Determination of the monoclonal antibody isotype may be accomplished by ELISA, thus providing additional information concerning purification or function.

Antibody fragments which contain specific binding sites of Del-1 may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to Del-1. Anti-Del-1

antibodies may be used to isolate Del-1-expressing cells or eliminate such cells from a cell mixture.

5.6. USES OF *DEL-1* POLYNUCLEOTIDE

A *del-1* polynucleotide may be used for diagnostic and/or therapeutic purposes. For diagnostic purposes, a *del-1* polynucleotide may be used to detect *del-1* gene expression or aberrant *del-1* gene expression in disease states. Included in the scope of the invention are oligonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes, that function to inhibit translation of *del-1*.

5.6.1. DIAGNOSTIC USES OF A *DEL-1* POLYNUCLEOTIDE

A *del-1* polynucleotide may have a number of uses for the diagnosis of diseases resulting from aberrant expression of *del-1*. For example, the *del-1* DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of *del-1* expression; *e.g.*, Southern or Northern analysis, including *in situ* hybridization assays. Such techniques are well known in the art, and are in fact the basis of many commercially available diagnostic kits.

5.6.2. THERAPEUTIC USES OF A *DEL-1* POLYNUCLEOTIDE

A *del-1* polynucleotide may be useful in the treatment of various abnormal conditions. By introducing gene sequences into cells, gene therapy can be used to treat conditions in which the cells do not proliferate or differentiate normally due to underexpression of normal *del-1* or expression of abnormal/inactive *del-1*. In some instances, the polynucleotide encoding a *del-1* is intended to replace or act in the place of a functionally deficient endogenous gene. Alternatively, abnormal conditions characterized by overproliferation can be treated using the gene therapy techniques described below.

Abnormal cellular proliferation is an important component of a variety of disease states. Recombinant gene therapy vectors, such as viral vectors, may be engineered to

express variant, signalling incompetent forms of Del-1 which may be used to inhibit the activity of the naturally occurring endogenous Del-1. A signalling incompetent form may be, for example, a truncated form of the protein that is lacking all or part of its signal transduction domain. Such a truncated form may participate in normal binding to a substrate but lack signal transduction activity. Thus recombinant gene therapy vectors may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of an Del-1. Accordingly, the invention provides a method of inhibiting the effects of signal transduction by an endogenous Del-1 protein in a cell comprising delivering a DNA molecule encoding a signalling incompetent form of the Del-1 protein to the cell so that the signalling incompetent Del-1 protein is produced in the cell and competes with the endogenous Del-1 protein for access to molecules in the Del-1 protein signalling pathway which activate or are activated by the endogenous Del-1 protein.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant Del-1 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing an *del-1* polynucleotide sequence. See, for example, the techniques described in Maniatis *et al.*, 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant Del-1 molecules can be reconstituted into liposomes for delivery to target cells.

Oligonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of a *del-1* mRNA are within the scope of the invention. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA,

oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between -10 and +10 regions of a *del-1* nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of *del-1* RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Methods for introducing polynucleotides into such cells or tissue include methods for *in vitro* introduction of polynucleotides such as the insertion of naked polynucleotide, *i.e.*, by injection into tissue, the introduction of a *del-1* polynucleotide in a cell *ex vivo*, *i.e.*, for use in autologous cell therapy, the use of a vector such as a virus, retrovirus, phage or plasmid, etc. or techniques such as electroporation which may be used *in vivo* or *ex vivo*.

5.7. USES OF *DEL-1* PROTEIN

Analysis of β-gal expression in transgenic mice in which β-gal gene expression is controlled by the *del-1* enhancer indicates that the *del-1* gene is activated in endothelial cells undergoing vasculogenesis. Vasculogenesis refers to the development of blood vessels *de novo* from embryonic precursor cells. The related process of angiogenesis is the process through which existing blood vessels arise by outgrowth from preexisting ones. Vasculogenesis is limited to the embryo while angiogenesis continues throughout life as a wound healing response or to increase oxygenation of chronically stressed tissues (Pardanaud et al., 1989 *Development* 105:473; Granger 1994, *Cell and Mol. Biol. Res.* 40:81).

It is likely that Del-1 functions during embryonic vasculogenesis and in angiogenesis. For therapeutic use, it is essential that Del-1, portions of Del-1 or antibodies that block Del-1, may interact with angiogenic cells since it is stimulation or inhibition of these cells that is clinically

relevant. Manipulation of Del-1 function may have significant effects on angiogenesis if Del-1 normally participates in this process.

The working examples in Sections 9 and 10 demonstrate that Del-1 exhibits an inhibitory effect on angiogenesis, which may be mediated by its interaction with $\alpha V\beta 3$-expressing endothelial cells. Del-1 protein or recombinant proteins consisting of portions of Del-1 may function to suppress angiogenesis or induce endothelial cell apoptosis. This function could be clinically useful to prevent neovascularization of tissues such as tumor nodules. It has been demonstrated that inhibition of angiogenesis is useful in preventing tumor metastases (Fidler and Ellis, 1994, *Cell* 79:185). Recently, O'Reilly et al (1994, *Cell* 79:315) reported that a novel angiogenesis inhibitor isolated from tumor-bearing mice, angiostatin, specifically inhibited endothelial cell proliferation. *In vivo*, angiostatin was a potent inhibitor of neovascularization and growth of tumor metastases. In a related report, Brooks et al (1994, *Cell* 79:115) showed that integrin antagonists promoted tumor regression by inducing apoptosis of angiogenic blood vessels. These integrin antagonists included cyclic peptides containing an RGD amino acid sequence. Since Del-1 contains an RGD sequence, the use of this portion of the Del-1 molecule may have similar effects.

Manipulation of the discoidin I/factor VIII-like domains of Del-1 may also be used to inhibit angiogenesis. Apolipoprotein E (ApoE) has been shown to inhibit basic fibroblast growth factor (bFGF)-stimulated proliferation of endothelial cells *in vitro* (Vogel et al., 1994, *J. Cell. Biochem.* 54:299). This effect could also be produced with synthetic peptides based on a portion of the ApoE sequence. These results could be due to direct competition of ApoE with growth factors for binding to heparin sulfate proteoglycans, or through disruption by ApoE of cell-matrix interactions. It has been proposed that discoidin I/factor VIII-like domains such as those in Del-1 bind to proteoglycans. In

addition, Del-1 is similar in structure to a number of extracellular matrix proteins. Thus, Del-1 may be manipulated to effect the activity of growth factors such as bFGF or to alter interactions between endothelial cells and the extracellular matrix.

The anti-angiogenic activity of Del-1 may be used to treat abnormal conditions that result from angiogenesis. These conditions include, but are not limited to, cancer, diabetic retinopathy, rheumatoid arthritis and endometriosis. Additionally, the removal or inhibition of Del-1 in situations where it naturally inhibits blood vessel formation may be used to promote angiogenesis. These conditions ~~inlcude~~ include, but are not limited to, cardiac ischemia, thrombotic stroke, ~~would~~ wound healing and peripheral vascular disease. Furthermore, Del-1 may be used to stimulate bone formation.

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6. EXAMPLE: MOLECULAR CLONING OF HUMAN AND MURINE *DEL-1* NUCLEOTIDE SEQUENCES

6.1. MATERIALS AND METHODS

6.1.1. GENERATION OF TRANSGENIC MICE

The SLM275 transgenic mouse line was generated in a C57BL6xDBA/F1 background, and the transgenic animals had been crossed back against similar B6D2F1 animals for maintenance of the line and the generation of embryos. This transgene had been maintained in the heterozygous state, and these heterozygous mice had normal breeding capacity. However, preliminary experiments indicated that these animals were not viable in the homozygous state.

6.1.2. MOLECULAR CLONING OF *DEL-1*

A genomic library was constructed from high molecular weight DNA isolated from the kidney of a SLM275 transgenic animal. This DNA was subjected to partial digestion with Sau3A to obtain an average size of 20 kb, subjected to a partial fill-in reaction, and then cloned into a similarly treated lambdaphage vector (lambdaFix,

Stratagene). The library constructed in this fashion had a base of approximately 2 million clones. These clones were amplified and the library stored at -70°C. A 200 basepair (bp) probe derived from the SV40 polyadenylation signal of the transgene was used as a probe and allowed the isolation of 12 lambdaphage clones. Six of these clones were randomly chosen for further investigation. These clones were mapped, and restriction fragments which did not contain transgene sequence identified. The clones were divided into two groups on the basis of common non-transgenic fragments. One such fragment from the first group of phage allowed specific hybridization to genomic blots and provided evidence that it was derived from a region adjacent to the integration site. Genomic DNA from a non-transgenic mouse of the same genetic background (B6D2F1) was compared to that of a SLM275 transgene animal by hybridization to this probe. Rearranged bands representing fragments disrupted by transgene integration were seen in the SLM275 lanes with both EcoR1 and BamH1 digests. The flanking sequence probe was employed to screen a commercially available lambdaFixII genomic library constructed from the 129SV mouse strain (Stratagene).

A murine cDNA fragment was used as a probe to identify cDNA clones of its human homolog. The probe corresponded to nucleotides 1249 through 1566 in the murine *del-1* major sequence. Human cDNA clones were isolated from a human fetal lung cDNA library (Clonetech, Inc.) following standard procedures.

6.2. RESULTS

A transgenic mouse line was created through a fortuitous enhancer trap event. The original studies were designed to map the cell-specific and developmental-specific regulatory regions of the mouse SPARC promoter, 2.2 kilobases (kb) of the SPARC 5' flanking sequence were placed upstream of the *E. coli lacZ* (beta-galactosidase or β-gal) reporter gene. The mouse SPARC gene is normally expressed in a wide variety of adult and embryonic cells which synthesize a specific

extracellular matrix (Nomura et al., 1989, *J. Biol. Chem.* 264:12201-12207). However, one of the founder mouse lines showed a highly restricted pattern of expression quite distinct from the native SPARC gene. Expression of the *lacZ* reporter in this particular line of mice referred to as SLM275 was seen very early in cells of the endothelial lineage. Whole mount *lacZ* staining was employed for initial studies, and these embryos were subsequently sectioned and examined by light microscopy. The first cells to stain were endothelial cells forming the endocardium, the outflow tract, and the developing intervertebral vessels. Staining appeared to be predominantly restricted to endothelial cells associated with forming major blood vessels. Expression began to decline after 11.5 days pc.

The genomic region targeted by this transgene is herein referred to as *del-1*. Initial cloning experiments were aimed at isolating genomic sequences flanking the transgene integration site. A number of lambdaphage clones were isolated and mapped (Figure 1). Approximately 40 kb of the wild-type *del-1* sequence was contained in these clones. By probing Southern blots containing restriction digests of these lambdaphages with non-transgenic fragments from the SLM275 lambdaphage clones, the site of transgene integration was mapped. Insertion of the transgene complex was associated with the deletion of approximately 8 kb of DNA. There were approximately 25 kb of flanking sequence on one side of the integration, and approximately 5 kb of the other flanking sequence contained on these clones.

Exon trapping was used to evaluate genomic fragments for the presence of exons. This approach utilized a vector with a constitutive promoter driving transcription through a DNA fragment containing a splice donor site and a splice acceptor site. Between these splicing signals was a common cloning site where the genomic DNA fragment to be evaluated was cloned. Exons within this fragment would be spliced into the transcript when the construct was transfected into eukaryotic cells, such as COS cells. The transcript containing the

trapped exon sequence was rescued from the COS cells by reverse transcriptase polymerase chain reaction (RT-PCR). PCR amplified DNA was cloned and evaluated.

A 160 bp exon was trapped from a fragment of genomic DNA located approximately 10 kb from the "left" integration site. Nucleotide sequence of the trapped exon was employed to screen various nucleic acid databanks through the BLAST routine at the NCBI, revealing no other gene with significant nucleic acid homology. The deduced amino acid sequence of the single open reading frame was subsequently employed in databank searches. These revealed that the protein domain encoded in the trapped exon was similar in part to domains in a number of proteins, including Factor V, Factor VIII and discoidin I (Figure 2) (SEQ ID NOS: 1-8) (Jenny et al., 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:4846-4850; Poole et al., 1981, *J. Mol. Biol.* 153:273-289; Toole et al., 1984, *Nature* 312:342-347). The protein which was most similar was milkfat globule protein, which had been found on the surface of mammary epithelial cells (1994, WO 94/11508). It has been hypothesized that the discoidin I-like domain in this protein allows it to localize to the surface of the epithelial cell (Larocca et al., 1991, *Cancer Res.* 51:4994-4998; Stubbs et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.* 87:8417-8421). The homologous regions of Factor V and Factor VIII have been implicated in their interaction with phospholipids on the surface of endothelial cells and platelets (Jenny et al., 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:4846-4850; Toole et al., 1984, *Nature* 312:342-347). Homology to the Xenopus protein A5 was also observed. A5 is a neuronal cell surface molecule which is expressed in retinal neurons and the neurons in the visual center with which the retinal neurons contact (Takagi et al., 1991, *Neuron* 7:295-307). A5 has been proposed to play a role as a neuronal recognition molecule in the development of this neural circuit, perhaps through mediating intercellular signaling. The protein for which this discoidin I-like domain was named is a protein expressed in *Dictyostelium*

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discoideum, which serves an essential role in the aggregation of individual cells.

The DNA fragment encoding the trapped exon was employed as a probe in a Southern blot experiment and shown to hybridize with regions of the *del-1* locus outside of the region that was employed in the exon trap construct. Given this finding, cDNA cloning was pursued by using the exon trap probe to screen an 11.5 day embryonic mouse cDNA library. Clones were plaque purified, and inserts subcloned into plasmid for further analysis. Nucleotide sequence analysis showed that two of the embryonic cDNA clones contained the sequence of the trapped exon. Sequence from the clones was used to expand the deduced amino acid sequence of the discoidin I-like domain (Figure 2) (SEQ ID NOS:1-8). The full nucleotide sequence of these cDNAs was analyzed and cloned into plasmid vectors which allowed the generation of cRNA transcripts for RNAse protection and in situ hybridization (Figure 3A-3E) (SEQ ID NO: 9).

A human cDNA was isolated from a human fetal lung cDNA lambdaphage library purchased from Clontech Inc. (Figure 4A-4C) (SEQ ID NO:11). A portion of the mouse *del-1* cDNA was used as a probe (Figure 5) (SEQ ID NO:19). The identity of the human cDNA clone was confirmed by comparing the human and mouse DNA sequences. These clones show approximately 80% DNA sequence homology and approximately 94% amino acid sequence homology (Figure 6) (SEQ ID NOS:10 and 50). These sequences are referred to as the "major" form of *del-1*. Upon initial isolation of *del-1*, standard molecular biology methods were used for isolating additional clones.

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DNA sequence analysis of the human *del-1* revealed an open reading frame of 1,446 base pairs predicted to encode a 481 amino acid protein with a molecular weight of 53,797. The mouse cDNA encodes a 480 amino acid protein. Homology comparisons with DNA and protein databases indicated that the Del-1 protein was composed of three EGF-like protein domains, followed by two discoidin I/factor VIII-like domains (Figure 7). Genes similar to *del-1* included some key regulators of cell determination and differentiation such as Notch. Overall, the Del-1 protein has a structure similar to the

membrane-associated milk fat globule membrane protein, MGF-E8, which has been used to develop antibodies for imaging breast cancer (Figure 8). amended 3.11.2002 (SEQ ID NOS: 20 and 21)

A physiologic function for the Del-1 protein is implicated by the activities which have been demonstrated for EGF-like and discoidin I/factor VIII-like domains in other proteins. EGF-like domains have been shown to participate in protein-protein binding interactions, while the discoidin I-like domains of factor VIII are believed to mediate binding to cell membranes through association with negatively charged phospholipids. Thus, the Del-1 protein may generate a signal for endothelial cell determination or differentiation by binding to the membranes of precursor cells and interacting with an EGF-like domain receptor protein.

Key structural features of the open reading frame of human Del-1 include:

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1) the presumed initiator methionine and putative secretion signal sequence (Figure 9) (SEQ ID NO: 22)
2) the three EGF-like domains (Figure 10) (SEQ ID NOS: 23-25)
3) the two discoidin I-like domains.

Further cloning and analysis of both the human and murine *del-1* genes revealed additional variant forms. For example, a human splicing variant (Z20 clone) was obtained in which 30 bp (i.e. 10 amino acids) (SEQ ID NO: 30, #66 - #75) between the first and second EGF-like domains of the major form (SEQ ID NO: 30) of *del-1* had been removed (Figure 11) (SEQ ID NO: 31). In addition, a truncated version of murine *del-1* was isolated, which contained a signal peptide sequence, all three EGF-like domains and only a partial amino-terminal discoidin I/factor VIII-like domain (about 40%). This variant is referred to as murine *del-1* minor sequence, which is disclosed in Figure 12A-~~12E~~ (12D, SEQ ID NOS: 28 and 29). This transcript was cloned only from mouse embryonic libraries, but was verified through cloning of several independent cDNAs.

7. EXAMPLE: TISSUE DISTRIBUTION OF *DEL-1* GENE EXPRESSION

7.1. MATERIALS AND METHODS

7.1.1. WHOLE MOUNT STAINING OF TRANSGENIC MOUSE EMBRYOS

Male transgenic animals of second or third generation had been crossed with 8-10 week B6D2F1 females, and embryos harvested at 7.5, 8.5, 9.5, 10.5, and 13.5 days. Timing was based on the convention that noon of the day of plugging was 0.5 day post-coitum (pc). Embryos were harvested, dissected free of decidua and membranes, fixed in 2% glutaraldehyde, and stained as a whole mount in a standard X-gal indicator solution according to standard protocols. An exception was that embryos older than 11.5 days were bisected which allowed better penetration of the fixative and staining solution. Stained tissues were identified in whole mount embryos by examination at 7-70x with an Olympus SZH10 stereomicroscope, and photographed under darkfield illumination. Embryos 7.5, 8.5, 9.5, and 13.5 days pc were embedded in paraffin, sectioned, counterstained with nuclear fast red and examined under brightfield with a Zeiss Axioplan microscope.

7.1.2. NORTHERN BLOT ANALYSIS

In order to study the expression of the *del-1* gene, Northern blots containing RNA obtained from a variety of human and mouse tissues (Clontech, Palo Alto, CA) were hybridized with a radiolabeled DNA probe as shown in Figure 5. SEQ ID NO:19. In addition, adult organs, 15.5 dpc whole embryos and organs dissected from embryos were disrupted with a polytron, and RNA isolated over CsCl ~~CsCl~~ gradient (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, NY). Briefly, the blots were prehybridized at 42°C for 3-6 hours in a solution containing 5X SSPE, 10X Denhardt's solution, 100 µg/ml freshly denatured, sheared salmon sperm DNA, 50% formamide (freshly deionized), and 2% SDS. The radiolabeled probe was heat denatured and added to the prehybridization mix and allowed to hybridize at 42°C for 18-24 hours with constant shaking.

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The blots were rinsed in 2X SSC, 0.05% SDS several times at room temperature before being transferred to a wash solution containing 0.1X SSC, 0.1% SDS and agitated at 50°C for 40 minutes. The blots were then covered with plastic wrap, mounted on Whatman paper and exposed to x-ray film at -70°C using an intensifying screen.

7.1.3 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated using standard laboratory procedures (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, NY). Approximately 1 μg of total RNA was reverse transcribed and the cDNA was amplified by PCR (Perkin Elmer, Norwalk, CT). The PCR amplification conditions were: 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec for a total of 40 cycles. The amplified products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The amplimers were:

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+ strand primer: ACC CAA GGG GCA AAA AGG A (SEQ ID NO:32)

- strand primer: CCT GTA ACC ATT GTG ACT G (SEQ ID NO:33)

7.2. RESULTS

Expression of *del-1* in various human and mouse tissues and cell lines was investigated by whole mount staining, Northern blot analysis and RT-PCR. Results of experiments are summarized in the subsections below.

7.2.1 EXPRESSION ANALYSIS BY HISTOCHEMISTRY

When the earliest time point was investigated by whole mount and histochemical staining in transgenic mice at day 7.5 pc, expression of the *lacZ* reporter gene was shown in cells forming the extra embryonic mesoderm (Figure 13A). These cells would form the yolk sac and give rise to cells of the blood island. Expression of the *lacZ* reporter gene in this locus is thus one of the earliest known markers of the endothelial cell lineage. The only other marker which has

been shown to be expressed in precursors of endothelial cells at this early stage of development is the receptor tyrosine kinase *flk-1* (Millauer et al., 1993, *Cell* 72:835-846). However, *del-1* expression was not found in the allantois, as with other early markers of the endothelium such as *flk-1* (Yamaguchi et al., 1993, *Development* 118:489-498).

At day 8.5, *lacZ* staining was seen in cells in the blood islands of the yolk sac. Interestingly, staining was not detected in mature endothelial cells lining the blood island, but rather in round cells found in clumps within the blood island (Figure 13B). These round cells had large nuclei and were closer in appearance to hematopoietic precursors rather than endothelial cells. This expression pattern was distinct from all other early endothelial markers. Thus, the *del-1* locus might be expressed in early embryonic cells which were precursors to both endothelial and hematopoietic lineages. In the late primitive streak stage embryo at 8.5 days pc, there was also staining of endothelial cells associated with the developing paired dorsal aortae. *LacZ* staining was seen in cells in the region of the forming heart at this stage, and these were presumably endothelial cells that would form the endocardium. By day 9.5 (10-14 somites), the endocardium and endothelial cells forming the outflow tract and aorta showed *lacZ* staining (Figure 13C, 13D). This staining persisted until day 10.5 and 11.5, and by whole mount analysis endothelial cells associated with all large vascular structures were expressing the reporter gene.

LacZ staining of embryos at day 13.5 of development was evaluated in the whole mount, and in sections made from paraffin embedded embryos. By this time, there was only patchy staining of endothelial cells in large vessels such as the aorta, whereas smaller vessels had virtually no staining (Figure 13E). The only blood vessels which showed prominent *lacZ* staining at this stage were the pulmonary capillaries. The developing pulmonary vascular network stained intensely, making the entire lung appear grossly blue-green (Figure 13E). Identification of the stained cells was made by

microscopy of stained sections (Figure 13F). Also, visualization of X-gal stained cells forming vascular channels was possible by viewing thick sections with Nomarski differential interference contrast optics. Organ vasculature associated the liver, brain and kidney showed no staining. In the heart, there was some residual staining of endothelial cells of the atrium. The majority of endothelial cells lining the ventricle no longer stained. The striking finding in the ventricle was that the cells forming the papillary muscle and the mitral valve showed marked staining. This labeling was seen not only in the endothelial cells on the surface, but in cells forming these structures. In a similar fashion, cells in the area of the forming valves of the aorta and pulmonary showed *lacZ* activity. Again, cells in the forming valve and in the wall of the vessel were stained (Figure 13G and 13H). The only non-cardiovascular staining was observed in cells in the areas of active bone formation. In particular, staining was most prominent in the proximal portions of the ribs, vertebrae, and the limb girdles (Figure 13E). After 13.5 days, the only cells expressing the *lacZ* gene were pulmonary endothelial cells. After approximately 15.5 days of development, expression of the reporter transgene diminished and was completely negative by the time of birth.

The aforementioned observations indicate that the protein encoded by the transcription unit in the *del-1* locus is involved in early developmental processes in the cardiovascular system. This gene is not only a lineage marker, since it is expressed in restricted groups of endothelial cells in a temporally regulated fashion. The restricted expression seen at later stages indicates a connection with the origin of these endothelial cells, the mechanism of blood vessel formation, or the context-derived phenotype of these cells. Cells of the primordial endocardium express this marker, indicating a role in cardiogenesis. Most striking is the pattern of expression in the developing valvular apparatus of the heart. Competent

endothelial cells in the forming septum and valves have been shown to undergo an epithelial-mesenchymal transformation. This transformation appears to be due, at least in part, to an inductive signal, such as transforming growth factor beta 3, which is released by the myocardium (Potts et al., 1991, *Proc. Natl. Acad. Sci. U.S.A.* 88:1516-1520; Sinning et al., 1992, *Anat. Rec.* 232:285-292). Reporter gene expression in the SLM275 mouse marked the competent cells of the endocardium which would respond to this signal, and expression appeared to persist for some time after the transformation (Figure 13G and 13H). This pattern of gene expression is unlike that described for any known molecule. Although the early endothelial expression pattern is similar to that characterized for the tyrosine kinases *tek* and *flk*-1 (Dumont et al., 1992, *Oncogene* 7:1471-1480; Millauer et al., 1993, *Cell* 72:835-846), there are striking differences at later stages which clearly indicate that *lacZ* expression in the transgenic animals marks a novel gene.

7.2.2. EXPRESSION ANALYSIS BY NORTHERN BLOT

Expression of *del-1* in various fetal and adult tissues was examined by Northern blot analysis (Tables 1 and 2). A portion of the mouse cDNA clone (0.3 kb Sac I probe) was used as a probe on six poly A RNA filters purchased from Clontech Inc. Human fetal tissues which were undergoing vasculogenesis were positive (Table 2). An organ blot generated with RNA from a 15.5 day mouse embryo indicated expression in highly vascular organs such as kidney, lung, nervous system and head. Also, the time course of expression in whole mouse embryos was consistent with the β-gal staining results observed in transgenic mice (Table 3). In general, adult mouse tissues were negative, or only weakly positive, (Table 4). Mouse cDNA clones isolated from a brain cDNA library appeared to be identical to the embryonic *del-1*. Two human cancer cell lines tested were weakly positive (Table 5). The results of Northern blot analysis were

basically consistent with the pattern for a gene which was specifically active during endothelial cell development.

Table 1

Human Adult

heart	+
brain	++++
placenta	-
lung	-
liver	-
spleen	-
thymus	-
prostate	-
testis	-
ovary	+
skeletal muscle	-
kidney	-
pancreas	-
small intestine	+
colon	-
peripheral blood leukocyte	+/-

Table 2

Human Fetal

brain	+++
lung	+++
liver	+
kidney	++
(Pooled from 17-26 wks)	

Table 3

Mouse Embryo

7-day	-
11-day	++
15-day	+++
17-day	++

Table 4

Mouse Adult

heart	-
brain	-
spleen	+
lung	-
liver	-
skeletal muscle	-
kidney	-

Table 5

Human Cancer Cell

Promyelocytic leukemia HL60	+/-
HeLa cell S3	+
chronic myelogenous leukemia K-562	-
lymphoblastic leukemia MOLT4	-
Burkit's lymphoma Raji	-
colorectal adenocarcinoma SW480	-
lung carcinoma A549	-
melanoma G361	-

7.2.3. EXPRESSION ANALYSIS BY RT-PCR

RNA from mouse yolk sac (day 8 through day 12) and mouse fetal liver (day 13 through day 18) were tested for *del-1* expression by RT-PCR. All tested samples were positive, consistent with the Northern blot analysis and results from β-gal staining in transgenic mice (Table 6). Several mouse yolk sac-derived cell lines were also tested by RT-PCR for expression of *del-1*. For comparison, several other cell lines and total d15 mouse fetal liver RNA samples were tested. All samples shown in Table 7 except ECV304 (a human endothelial cell line) were of mouse origin. The yolk sac-derived cell lines grown in long-term culture were not expressing *del-1* at a detectable level. These cell cultures were not forming endothelial cell-like structures under these conditions. In contrast, an endothelial tumor line, EOMA, expressed high levels of *del-1*.

Table 6

Yolk Sac and Fetal Liver

Sample	Result
d8 Yolk Sac	+
d9 Yolk Sac	+
d10 Yolk Sac	+
d11 Yolk Sac	+
d12 Yolk Sac	+
d13 Fetal Liver	+
d14 Fetal Liver	+
d15 Fetal Liver	+
d16 Fetal Liver	+
d17 Fetal Liver	+
d18 Fetal Liver	+

Table 7

Cell Lines

cell line	*del-1*
3T3 A31	-
Sto 1	++
YS4	-
Pro135	-
Pro175	-
D-1	-
A10	-
ROSA02	-
dl5FL	++
EOMA	+++
ECV304 (human)	-

A number of human tumors implanted in nude mice and cultured *in vitro* were shown to express *del-1* by RT-PCR. For example, Table 8 shows the expression of *del-1* in human osteosarcoma cell line 143B *in vivo* and *in vitro*. EOMA was used as a positive control. CD34, *flk-1* and *tie-2* are known markers for endothelial cells. When human and mouse *del-1* specific PCR primers were used, both human (tumor) and murine (host) *del-1* expression was detected. In addition, a variety of human tumor cell lines expressed *del-1* in culture (Table 9). These results indicate that Del-1 may be used as a tumor marker in certain cancers diagnostically and therapeutically. In addition, host expression of *del-1* is also up-regulated, possibly due to angiogenesis in tumor sites.

Table 8

Human osteosarcoma 143B

Sample	Actin	*del-1*	CD34	*flk-1*	*tie-2*
control nude mouse skin	-	-	nd	nd	nd
7 day tumor	+	+	nd	nd	nd
10 day tumor	+	+	+	+	+
14 day tumor	+	+	+	+	+
cultured 143B cells	+	+	-	-	-
EOMA	+	+	+	+	+

nd = not determined

Table 9

Human tumor cell lines

Cell Type	Sample	27 cycles	33 cycles
Normal	Myoblast	+	+++
	HYSE-E	+	+++
	HYS-VS1	++	++++
Leukemia	K562	-	-
	HEL	-	+/-
	Mo7e	-	-
Glioblastoma	U-118 MG	+	+++
	U-87 MG	++	+++
CNS Tumor	SF295	+	+++
	U251	++	++++
	SNB75	++	++++
	SNB19	+	+++
	SF539	+	+++
Osteosarcoma	143B	+	++++
Breast Carcinoma	DU4475	-	-
	MCF-7	+/-	+++
	MDA231	+	+++
Endothelial	ECV304	-	-
	HUVEC	+ -	+++

8. EXAMPLE: IMMUNOREACTIVITY OF DEL-1 GENE PRODUCT

8.1. MATERIALS AND METHODS

8.1.1. ANTIBODY PRODUCTION

A partial *del-1* cDNA encoding amino acids 353 to 439 of the murine gene was cloned into pMALC2 (New England Biolabs) to generate a maltose binding protein-partial Del-1 fusion protein. The *del-1* sequence included in this construct encodes a portion of the carboxyl terminal discoidin-like domain. Recombinant fusion protein was expressed and purified over an amylose affinity matrix according to the manufacturer's recommendations. Protein was emulsified into Freund's complete adjuvant, and injected as multiple subcutaneous injections into two New Zealand White rabbits. Boosting and harvesting of immune serum was performed according to established methodology (Harlow and Lane, 1988, Antibody: A Laboratory Manual, Cold Spring Harbor Laboratory). Immune serum obtained after the second boost was subjected to affinity purification. First, the antiserum was precleared over a Sepharose column coupled to total bacterial lysate. Subsequently, the antiserum was purified over an affinity column made from recombinant fusion protein coupled to Sepharose. The specificity of the antiserum was evaluated first with western blots containing proteins from bacteria expressing the recombinant fusion protein before and after cleavage with factor Xa, or the maltose binding protein alone. Whole bacterial lysates from cells induced with IPTG were run on polyacrylamide gels, transferred to nitrocellulose, and probed with the affinity-purified antiserum. While crude antiserum labeled bands corresponding to maltose binding protein and the Del-1 portion of the fusion protein, affinity-purified antiserum specifically labeled the Del-1 component of the fusion protein.

8.1.2. WESTERN BLOT

For western blots of eukaryotic proteins, cells were harvested by lysis in a standard lysis buffer or Laemmli loading buffer. Cell culture supernatant was collected and

concentrated by centrifugation in a centricon filter, and extracellular matrix harvested by first removing cells with 1 mM EDTA in PBS, and then scraping the cell culture dish with a small volume of Laemmli buffer at 90°C.

8.1.3. IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on sections prepared from Bouin's fixed, paraffin-embedded, staged mouse embryos according to well established methodology (Hogan et al., 1994, *Manipulating the Mouse Embryo*, Cold Spring Harbor Press; Quertermous et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:7066). The affinity-purified Del-1 antiserum was employed at a dilution of 1:500 to 1:1000, and the specificity of staining verified by competition with recombinant protein. Staining of cartilage was amplified by pre-treating the section with dilute trypsin solution.

8.1.4. TRANSFECTION OF YOLK SAC CELLS

A eukaryotic expression vector was constructed by cloning the entire open reading frame of the major *del-1* transcript into phbAPr-3-neo (Gunning et al., 1987, *Proc. Natl. Acad. Sci. USA* 84:4831). This construct was transfected into yolk sac cells with Lipofectamine (Gibco BRL), and clones selected in the presence of 1000 μg/ml of G418. Clones were evaluated for *del-1* expression by northern and western blotting, and a group of clones with varying amounts of Del-1 protein were selected for further study. To serve as negative controls, a group of clones were randomly selected from a transfection with the empty phbAPr-3-neo vector.

8.2. RESULTS

The major murine *del-1* coding sequence was inserted into a eukaryotic expression vector and transfected into Del-1-non-expressing yolk sac cells (Wei et al., 1995, Stem Cell 13:541). Pooled transfectants with an empty expression vector or the *del-1* construct were selected in G418.

Lysates, cell culture supernatants and extracellular matrix were prepared from transfected cells, and reacted with an affinity-purified rabbit antiserum in Western blots. The polyclonal antiserum was generated to recombinant Del-1 fusion protein expressed in bacteria. Figure 14A shows that a band of 52,000 daltons molecular weight was recognized in cell lysates prepared by harvesting the cells in lysis or standard Laemmli gel loading buffer, and in extracellular matrix. This band corresponds with the predicted molecular weight for Del-1 based on the deduced amino acid sequence, and represented the full-length Del-1 protein. In contrast, no protein was identified with culture supernatants harvested from the transfectants, even when concentrated 100-fold. Additionally, smaller proteolytic fragments were also detected. These results indicate that Del-1 is secreted across the surface of endothelial cells, and deposited in the extracellular matrix.

Several stably transfected yolk sac cell clones with the *del-1* gene were selected (Figure 14B). When the transfected cells were reacted with the aforementioned antibody, both the membrane of certain yolk sac cells and the extracellular matrix were stained as compared with mock-transfected yolk sac cells as negative control (Figure 15A, 15B). In keeping with this staining pattern, immunostaining of developing bone of a 13.5 day mouse embryo detected the Del-1 protein in the ~~laquanae~~ lacunae within the bone, which were composed of extracellular matrix proteins (Figure 16).

In order to test the expression of *del-1* in tumor cells by immunohistochemistry, human glioma cells were implanted in nude mice. The tumor was isolated, sectioned and stained with the aforementioned antibody followed by an anti-rabbit antibody conjugated with horse radish peroxidase and developed with Sigma Fast Red substitute. Figure 17A shows that the *in vivo* tumor cells were stained with the antibody in a polarized fashion. Polarization of *del-1* expression in tumor cells might have resulted from the interaction of the gene product with cellular receptors on adjacent cells. In

addition, a blood vessel of mouse origin traversing the human tumor was also stained with the antibody (Figure 17B).

9. EXAMPLE: DEL-1 INHIBITS VASCULAR FORMATION

9.1 MATERIALS AND METHODS

9.1.1. ANGIOGENESIS ASSAYS

In vitro angiogenesis assays on "MATRIGEL" (Biocoat, Becton Dickinson) were conducted in 24 well plates coated with 50 μl of "MATRIGEL". *del-1* transfectants and control transfectants were plated at a density of $5x10^4$ cells/well (low density) or $2x10^5$ cells/well (high density), and observed for seven days.

For the assay evaluating morphogenetic potential of wild type yolk sac cells on *del-1* conditioned matrix, the matrix was generated by growing 10^5 *del-1* transfectants in 6 cm dishes for 7 days. A control matrix was generated by growing control transfectants under identical conditions. Transfected cells were removed with 0.5 M EDTA and extensive washing, and 10^5 wild type yolk sac cells were plated on the matrix produced by the *del-1* or the control transfectants. Cells were cultured and observed for seven days.

For the *in vitro* angiogenesis sprouting assay, *del-1* and control transfectants were trypsinized, and 10^5 cells cultured in a 15 ml conical tubes for 48 hours. Cell cultures were then transferred into a bacterial petri dish, and cultured for 4-7 days. Under these conditions, cell aggregates were formed. Several aggregates were collected for *del-1* and control transfectants, and these were transferred to 24 well plates coated with "Matrigel". Sprouting angiogenesis was evaluated at 24 and 48 hours.

9.2. RESULTS

The yolk sac cell line, YS-B, was chosen as the parental cell for *del-1* transfection because it had characteristics of embryonic endothelial cells, did not express *del-1*, was clonal and long lived in culture (Figure 18A). Most importantly, these cells provided a model of vascularization

of the early yolk sac. While they were easily grown and maintained with frequent passage, when allowed to accumulate to high density they spontaneously formed vascular structures. This process was accelerated when the cells were plated on the basement membrane-like material "MATRIGEL", on which they behaved similar to various types of cultured endothelial cells (Figure 18B). Cell lines transfected with the cDNA encoding of the major form of *del-1* were selected for varying levels of expression of the transfected construct (Figure 14B). Cell lines transfected with the empty expression plasmid were selected to serve as negative controls.

The *del-1* transfected yolk sac clones and mock-transfected yolk sac lines were compared for their ability to form branching vascular-like structures on "MATRIGEL". After 24 hours on "MATRIGEL", the negative control transfectants had established an intricate network typical for these cells (Figure 18C). Cells (L10) expressing high levels of *del-1* showed a markedly different pattern, assembling into multiple well-spaced clusters (Figure 18D). This abrogation of morphogenesis was directly related to the level of *del-1* expression, as low *del-1* expressing clones, L13 and L14, showed some degree of branching morphology.

Since Del-1 protein is deposited in the extracellular matrix, one *del-1* expressing clone, L10, was used to generate a cell culture matrix containing Del-1 protein. Matrix generated by negative control clones should differ only by the absence of Del-1. Transfected and control lines were cultured for 7 days, and then gently removed from the culture dish by extensive washing with 1 mM EDTA. By visual inspection, only a rare cell was not removed with this technique. Non-transfected native yolk sac cells were then plated on the Del-1-containing and the control matrices, and scored for their ability to assemble into a network. The yolk sac cells required several days at high density to undergo morphogenesis, and the network was lace-like in appearance. Cells grown on the matrix produced by negative

control transfectants were able to produce the network (Figure 18E). In contrast, yolk sac cells grown on matrix containing Del-1 revealed no evidence of morphogenesis. They formed instead a dense monolayer (Figure 18F).

Next, an *in vitro* angiogenesis sprouting assay was employed with the transfected yolk sac lines. This assay has been employed to evaluate angiogenic potential (Pepper at al. 1991, J. Cell. Physiol. 146:170). Transfected cells were allowed to stand overnight in a conical tube to allow them to aggregate, and the cell mass was then placed on "MATRIGEL". The ability of the *del-1* expressing cells to migrate onto the "MATRIGEL" and assemble into branching structures was compared to control cells. Within 24 hours, the control cells formed a series of branching projections, while the cells expressing *del-1* remained virtually confined to the cellular aggregate (Figure 18G and 18H). While there was some evidence of spreading of the *del-1* expressing cells after 48 hours, it was more as a sheet rather than a sprouting structure.

Hence, Del-1 inhibits vascular morphogenesis and may be used to regulate endothelial cell differentiation.

10. EXAMPLE: DEL-1 BINDS TO INTEGRIN ALPHA V BETA 3

10.1. MATERIALS AND METHODS

10.1.1. RECOMBINANT DEL-1 PURIFICATION AND REFOLDING

Recombinant murine Del-1 protein (major form) was prepared using an *E. coli* expression system and protein refolding technique. *E. coli* cells with the *del-1* containing pET28a vector (Novagen Inc.) were grown and induced following the protocol recommended by the manufacturer. Approximately 50 to 100 mg of crude recombinant Del-1 were routinely produced from 1L of bacterial culture in the form of insoluble cytoplasmic inclusion bodies. Inclusion bodies were isolated by sonication of the *E. coli* cells, centrifugation and collection of the pellet fraction.

Inclusion bodies from 500 ml of culture were then washed three times with 50 ml of 2M Urea, 0.025 M Tris-Cl (pH8.0), 0.025% Triton X100. This procedure yielded a crude, insoluble, Del-1 product of > 80% purity.

Recombinant Del-1 was dissolved by suspending the pellet from 500 ml of culture in 2.5 ml of 8M Urea, 100 mM DTT, 0.1 M Tris-Cl (pH8.0), 0.05% Triton X100, followed by incubation at room temperature for 1 hr. Insoluble material remaining was removed by centrifugation and the soluble supernatant fraction was diluted 10 fold to 25 ml with 8M Urea, 100 mM Tris-Cl (pH 8.0), 0.05% Triton X100. Protein concentration was then measured by Bradford protein determination assay.

Soluble, reduced Del-1 was refolded by diluting to a final concentration of 0.01 mg/ml into refolding buffer: 100 mM Tris-Cl (pH8.0), 100 mM $(NH_4)_2SO_4$, 2 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.05% sodium azide, 0.025 mg/ml PMSF. Refolding was performed by incubating this reaction mix at 4°C for one week. Refolded Del-1 was then concentrated using an Amicon spiral concentrator and the soluble material remaining was collected.

The recombinant Del-1 product produced from the pET28a expression vector is a fusion protein with both N-terminal and C-terminal polyhistidine tags. This product was purified using the Novagen His tag resin purification system, following the protocol recommended by the supplier.

Refolded murine recombinant Del-1 was soluble and stable when stored at 4°C in Tris-Cl buffer with 100 mM $(NH_4)_2SO_4$ at concentrations of less than or equal to 100 mg/ml.

10.1.2. CELL ADHESION ASSAYS

Human umbilical vein endothelial cells (HUVEC) (Clonetics Inc., San Diego, CA) were grown as indicated by the supplier in endothelial growth media supplemented with 10 ng/ml human recombinant epidermal growth factor, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 12 µg/ml bovine brain extract and 2% FBS. Cells were grown at 37°C/5% CO_2 to 70% confluency before use in the binding assay. Non-tissue

culture treated 96 well plates were coated with appropriate levels of target protein (1-20 μg of either murine recombinant Del-1, vitronectin, or BSA) diluted in calcium and magnesium free PBS for 24 hrs at 4°C. The plates were washed with PBS and blocked for 30 min with a solution of heat treated (95°C for 5 min) PBS containing 3% BSA. HUVEC cells were harvested by trypsinization and resuspended in an adhesion buffer (Hanks balanced salt solution pH 7.4 containing 10mM Hepes, 2.2 mM $MgCl_2$, 2 mM $Cacl_2$, 0.2mM $MnCl_2$ and 1% BSA). Cells (10^4/100 μl) were added to each well in the presence or absence of the indicated antagonists or controls at varying concentrations. Antagonists included anti-human αVβ3 (clone LM609, Chemicon Inc.), RGE peptides (the inactive control GRGESP) or RGD the stable antagonist GPenGRGDSPCA or GRGDdSP all from Gibco). Cells were incubated at 37°C/5% CO_2 for 60-90 min and wells were washed until no cells remained in the BSA control. To count remaining cells, 100 μl of endothelial media was added to each well. Cells number was determined by the Promega Cell titer AQ as indicated by the manufacturer.

10.2. RESULTS

Recombinant Del-1 protein and *del-1* transfectants bound HUVEC. In order to identify a cellular receptor on HUVEC for Del-1, various peptides and antibodies were used to inhibit the interactions between Del-1 and HUVEC in cell adhesion assays. Figure 19 shows that an anti-αVβ3 antibody specifically inhibited recombinant Del-1 binding to HUVEC. In contrast, anti-αVβ5 did not inhibit, nor did the control Ig. Furthermore, an RGD peptide was also shown to inhibit Del-1 binding to HUVEC (Figure 20). Similar results were obtained using extracellular matrix obtained from *del-1* transfected cells. Therefore, Del-1 binds to αVβ3 expressed by HUVEC, possibly via RGD in its second EGF-like domain.

αVβ3 is an integrin expressed by certain cell types and is associated with bFGF-induced angiogenic endothelial cells. Agents that bind to this integrin induce apoptosis of

angiogenic endothelial cells. Since Del-1 binds to this integrin, it may be used to induce apoptosis during angiogenesis in tumor sites to reduce tumor growth.

11. EXAMPLE: CHROMOSOMAL LOCALIZATION OF HUMAN *DEL-1*

DNA from P1 clone 10043 was labeled with digoxigenin dUTP by nick translation. The labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2X SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI. The initial experiment resulted in specific labeling of the long arm of a group B chromosome.

A second experiment was conducted in which a probe that had previously been mapped to 5q34, and confirmed by cohybridization with a probe from the cri du chat locus which is known to localize to 5p15, was cohybridized with clone 10043. This experiment resulted in the specific labeling of the mid and distal long arm of chromosome 5 (Figure 21 A and B). Measurements of 10 specifically hybridized chromosomes 5 demonstrated that clone 10043 was located at a position which was 29% of the distance ~~form~~ from the centromere to the telomere of chromosome arm 5q, an area that corresponded to band 5q14. A total of 80 metaphase cells were analyzed with 74 exhibiting specific labeling. This region of the chromosome has been found to be a break point in some human cancers (Wieland and Bohm, 1994, *Cancer Res.* 54:1772; Fong et al., 1995, *Cancer Res.* 55:220; Wieland et al., 1996, 12:97, *Oncogene* 12:97). Thus, chromosome 5 aberrations may lead to altered expression of *del-1* and contribute to the malignant phenotype.

12. DEPOSIT OF MICROORGANISMS

The following organisms were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852.

Strain Designation	Accession No.
Hu DEL-1.Z1	ATCC 97155
Hu DEL-1.Z20	ATCC 97154
mus DEL-1.1	ATCC 97196
mus DEL-1.18	ATCC 97197

The present invention is not to be limited in scope by the exemplified embodiments or deposited organisms which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

All publications cited herein are incorporated by reference in their entirety.